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Anne Holleran
AU: 1642
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mailbox: 8e12

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Immunogenicity of tumour associated antigens

SHAHID MIAN, R. ADRIAN ROBINS, ROBERT C. REES and BERNIE FOX

INTRODUCTION

The continuing discovery of new tumor-associated/specific antigens, many of which are discussed in succeeding chapters in the first half of this book, document that at least some (most?) types of cancer are antigenic. Within the past five years, the number of molecular and cellular immunological techniques for identifying tumour-associated antigens has increased to such extent that over 100 distinct genes have now been associated with the transformation process. These antigens have been classified into several sub-groups and include for example proteins that are either mutated [1, 2], over-expressed [3, 4], associated with embryo-genesis [5] or differentiation [6]. They also include novel products that arise due to genetic translocations such as BCR-Abl [7, 8]. Melanoma is particularly interesting from an immunological perspective because it contains a wide spectrum of tissue-restricted proteins (e.g., MART-1, MAGE, gp100, tyrosinase, TRP-1, and TRP-2) that serve as targets of effector T cells in vitro [9-13]; see also Chapters 3 and 4. However, in vivo, adequate spontaneous activation of tumor-specific lymphocytes either does not occur or it results in inefficient tumor protection. Why tumors that are clearly antigenic are so clearly nonimmunogenic has puzzled investigators for years. Aspects of this paradox will be considered in this introductory chapter, including tolerance, antigen processing and the role of dendritic cells, and the nature of the response induced in terms of the balance between cellular immunity (Th1 type response) and antibody responses (Th2 type response).

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TOLERANCE

Burnett and Fenner have defined tolerance rather simply as "non-reactivity against self". If most tumor antigens are indeed self antigens, and if the tumor-bearing host must ensure "non-reactivity against self," then the host seems doomed to succumb to their cancer. This is what happens all too often to cancer patients. A broader appreciation of tolerance in the tumor-bearing host and how it affects the immune response to tumor antigens is essential to the development of therapeutic cancer vaccines. Our understanding of immunological tolerance has changed dramatically over the past decade. We now recognize a number of measures the host uses to induce tolerance and protect itself from the induction of auto-aggressive T cells. High affinity, auto-reactive T cells may be deleted centrally in the thymus while other non-deletional pathways are available for induction of tolerance in the periphery [reviewed in 14]. Functional silencing (or anergy) can occur as a consequence of T-cell stimulation by antigen and MHC in the absence of costimulation [15, 16]. Antigen-specific immune responses occur in models of tolerance, and appear to be responsible for tolerance induction by developing a non-destructive rather than a destructive immune response, a mechanism referred to as immune deviation [reviewed in 16]. These findings are consistent with the definition of tolerance proposed by Schwartz, "a physiologic state in which the immune system does not react destructively against self." Thus, the production of inefficient immune responses that are not capable of reacting destructively against self may be responsible for the poor results associated with many immunotherapies.

ANTIGEN PROCESSING

Introduction

As with all cellular proteins, tumour associated antigens are processed and degraded to 8-10 amino residue peptides via the 26S proteasome complex. The peptides are transported across the ER membrane in association with chaperone proteins and TAP where they are complexed with MHC class I for cell surface presentation [reviewed by17]. By priming the immune system to recognise cells expressing particular epitope/MHC configurations, these complexes have been shown to act as specific molecular targets for CTL mediated killing [6, 18, 19]. A fundamental and requisite component in eliciting this recognition/killing pathway against tumour cells is the activation of professional antigen presenting cells (APCs). Although B-cells and macrophages are members of this group, it is the dendritic cell that has emerged as the most potent stimulator of immune effector functions in relation to cancer immunotherapy [20].

Dendritic Cells

Dendritic cells have been divided into three functional classes that are based primarily upon tissue distribution. Langerhan's cells (LC) for example are found either within the skin or organs possessing mucosal linings [e.g. lungs and nasal passages;21]. Interstitial DCs reside within deeper tissues such as the liver, kidney or heart, while activated/"mature" DCs constitute the final group and represent a class of cells that have altered biochemical and physical properties to those of either the Langerhan or interstitial category [20]. "Immature" cells are known to have an excellent ability to take up antigens from the external medium, but are poor at presenting processed antigens to T-cells, whereas the reverse is true for "mature" DCs. In order to understand how DCs stimulate potent anti-tumour responses via CTL, it is necessary to understand firstly how tumour antigens are processed and presented to the immune system and secondly how accessory receptors are needed to obtain maximal T-cell activation. A summary of how DCs have been used in the clinical treatment of cancer to date will then follow.

Antigen Uptake

To fulfil their role within antigen presentation, it is an obligatory requirement for immature DCs to present antigens that have been derived from both endogenous and exogenous sources, however, it is the latter route that is probably the most common form in which a DC will encounter an antigen from a tumour cell. Endogenous and exogenous antigens are normally presented via MHC class I and class II respectively [reviewed by22], however, for externally derived antigens DCs have evolved two major mechanisms for internalising macromolecules. The first involves macro-pinocytosis and serves to endow the DC not only with an ability to sample large volumes of the external milieu and thus increase the change of coming into contact with a foreign/tumour antigen, but also to enable large soluble molecules to be internalised and presented to the immune system [23, 24]. The second mechanism involves receptor-mediated endocytosis and includes for example both the mannose [23] and Fc cell surface receptors. For proteins glycosylated with mannose, presentation of antigens was reported to be 100 fold higher, if internalisation occurred via mannose-receptor mediated endocytosis [24]. It was also suggested that the system might even function to concentrate antigens (present in low concentrations) on the cell surface [25]. This might in part explain why peptides covalently linked to mannose resulted in 100-10,000 fold greater stimulation of T-cells when compared to the same peptide devoid of sugar residues [26]. The mannose receptor is believed to be a prototypic member of a new family of proteins that recognise the "sugar pattern" coating antigens from foreign agents [27]. It is believed that these pattern recognition receptors are linked to signal transduction pathways and promote the release of cytokines when appropriately stimulated. The mannose-receptor system however, is not universally used by all DC lineages as exemplified by LC. In this scenario, it has been suggested that a reduced rate of antigen uptake/presentation may of benefit to the host, by limiting immune responses to antigens that are encountered frequently within the skin [21]. Immuno-histochemisty data would also suggest that MHC class II molecules and mannose-receptors in fact

reside within separate sub-cellular compartments and that the direct transfer of antigens from receptor to MHC class II is unlikely [26, 28, 29].

In contrast to the mannose receptor system, Fc receptors are a group of membrane bound proteins that bind the Fc portion of immunoglobulins such as IgE or IgG and consequently internalise antigens indirectly [30, 31]. Although antigen binding to MHC class I and class II molecules occurs through independent routes, "cross-priming" is an apparent mechanism in which exogenous antigens can be directed into the class I loading pathway. This is known to occur for antigens that are taken up via the Fc gamma R receptor and is dependent upon the both the proteolytic activity of the 26S proteasome and ER transportation involving TAP1/TAP2 [31]. The cross-priming phenomenon is not restricted to receptor-mediated mechanisms. For instance MHC class I presentation of antigens has also been reported for proteins taken up by macropinocytosis. In this pathway, antigen presentation was also contingent upon functional 26S proteasome and TAP transporter proteins [32]. Although discrepancies have existed between in-vitro experiments as to whether cross priming does indeed require the presence of the TAP transporters, Huang et al, have reported an absolute requirement for these proteins in-vivo [33].

Recent evidence is emerging about the existence of a third receptor system involved in antigen uptake and presentation upon MHC class I. DCs have been shown to phagocytose vesicles from apoptotic cells and direct antigen processing towards the class I pathway [34]. The ability to phagocytose apoptotic cells is restricted to immature DCs that express a defined set of surface membrane receptors including for example, the alpha v beta5 integrin and CD36. Upon maturation these two receptors are specifically down regulated and the rate of phagocytosis is reduced. Macrophages are equally adept at phagocytosing apoptotic cells however, they are incapable of presenting antigens via cross priming mechanisms and it has been suggested that this unique ability of DCs is contingent upon alpha v beta5 integrin expression [35]. The inability of macrophages to cross prime antigens derived from exogenous sources was also reported by Ronchetti and colleagues [36]. Antigens that are presented via MHC class I due to cross priming have the ability to actively stimulate $CD8^+$ T-cells [37]. One possible explanation of how cross priming may occur is that some MHC class I molecules have the ability to enter acidic MHC class II containing compartments. Peptides generated from the degradation of exogenously derived proteins would be able to directly load onto MHC class I before trafficking to the cell surface [38].

Sources of Antigen

The need for APCs to evolve multiple types of receptor and non receptor-mediated pathways for antigen uptake and presentation has resulted primarily in response to the heterogeneity of antigens. For example a single protein antigen can be presented in a variety forms ranging from a simple unmodified form to having a complex array of glycosylated residues attached to the protein backbone. It is also possible that proteins could be presented to dendritic cells not in isolation, but as a complex with other proteins e.g. the heat shock protein family of chaperones. We have also seen that macromolecular structures such as apoptotic bodies bind to specific receptors on

dendritic cells before they are engulfed and processed for antigen presentation. As a consequence therefore, the ability of a dendritic cell to mount an effective immune response against a given antigen is contingent not only upon mechanisms in place that will process an antigen regardless of the form in which it is presented, but also in having the capacity to "prioritise" or "rank" antigen potency and to use this as a measure of whether an immune response should be elicited or not. For example antigens that are presented in the form of apoptotic bodies and whose immunogeneicity is low can be made immunogenic by presenting the same apoptotic bodies in the presence of over-expressed HSPs. The same pattern has been demonstrated for peptides coated with mannose residues, in which presentation to T-cells leads to greater stimulation than those that are not. This could suggest that host antigens are often presented to DCs using particular receptor pathways and that these pathways are attenuated so as to prevent improper activation of immune responses against host derived antigens.

MATURATION OF DENDRITIC CELLS IN RESPONSE TO ANTIGEN STIMULATION

Although DCs represent the most potent activators of cell mediated immunity they do however constitute a sub-population of APCs that are present in relatively fewer numbers than their macrophage or B-cell counterparts. In addition, they are also known to be widely distributed throughout tissues which makes them difficult to isolate in large enough numbers for scientific study. To circumvent these problems, DCs have been generated in-vitro using one of either two-precursor cell sources. These include both $CD34^+$ bone marrow stem cells and circulating blood monocytes [39]. In the non-activated or "immature" state, DCs have an extreme proficiency for taking in and degrading exogenously derived antigens however, in order to stimulate a specific immune response towards these antigens it is mandatory for the cell to "mature". The process of maturation is tightly regulated and associated with a specific alteration in function that involves the cell down regulating its ability to engulf/process antigens and up regulating its antigen presentation capacity [39]. There is also a concomitant reduction in MHC class II containing intracellular compartments and an increase in expression of a well-defined set of cell surface markers. These include for example CD1, CD83, Ox40L, CD40, CD80 (B7.1), CD86 (B7.2), as well as an up-regulation of MHC class I and MHC class II [39, 40]. In-vitro DC maturation has been achieved through the use of a number of cytokine "cocktails" depending upon the progenitor cell. Although bone marrow derived DCs have been used in experimental systems for studying a diverse range of immunological functions, human gene therapy experiments have initially concentrated upon using monocyte derived DCs due to the ease of isolating large numbers of cells (discussed below). Monocytes incubated in the presence of GM-CSF and IL-13 for several days are known to alter their morphology/cytology and adopt DC like characteristics including the expression of CD1a and increased MHC trafficking to the plasma membrane [41].

Maturation Stimuli

To gain further understanding of the maturation process and to obtain maximal $CD4^+$ T-cell stimulation in response to antigen priming Cella and colleagues conducted studies that assessed the expression kinetics of MHC class II in both immature and mature DC. It was observed that in the immature state, DCs were constantly recycling MHC class II from the surface into highly acidic intracellular endosome type compartments. The half-life of membrane bound MHC class II molecules was noted to be approximately 10 hours. However exposure to inflammatory stimuli resulted not only in an increased rate of gene expression for MHC class II but it also led to a half-life extension of 100 hours for surface bound molecules [42]. DCs are extremely proficient at presenting antigens to T-cells from macromolecular structures such as viruses. In a specific example involving the influenza virus, DC maturation was stimulated both as a direct response to viral infection and as a result of the presence of double stranded RNA. Type I interferon and MxA were shown to be directly up regulated in response to infection and it was suggested a combinatorial effect had occurred that enabled the DC not only to resist the cytopathic effect of the virus (and hence prevent its early demise) but also to augment its overall antigen presenting capabilities [43].

DCs can also be activated in response to "normal" physiological stimuli such as the deletion of cells via apoptosis. This form of programmed cell death is considered the usual route in which cells are removed from tissues upon completing their lifespan [44-46]. There is now an accumulation of evidence suggesting that DCs are capable of taking up apoptotic vesicles through a specific set of receptors [34] and presenting antigens derived from them directly to either $CD8^+$ [35] or $CD4^+$ T-cells. A number of studies have been conducted to assess the relative immunogenicity of apoptotic bodies taken up by DCs and current data would suggest that apoptotic bodies are in fact poor immune activators of DCs [36]. Activation of DCs could only be achieved in the presence of large numbers of apoptotic bodies suggesting that the degree of activation was directly proportional to the number of cells undergoing apoptosis [47]. From a physiological point of view therefore, these data lead to the hypothesis that DCs are unable to activate immune responses against tissues undergoing normal cellular turnover as they produce only low numbers of "poorly" immunogenic apoptotic bodies. An accumulation of these apoptotic bodies above a normal physiological threshold would consequently lead to an effective stimulation of DCs and thus alert the immune system to perturbations occurring in cellular turnover. Maturation of DCs in response to an apoptotic stimulus would result in the presentation of host tissue antigens to the immune system in the absence of activating inflammatory stimuli or "danger signals" [47].

"Danger signals"

The idea of "danger signals" was first developed by Matzinger who suggested that the immune system does not actually discriminate between self and non-self antigens but instead responds to inflammatory stimuli as a means of detecting abnormalities (e.g. microbial infections) within the host [48]. This hypothesis has begun to gain a huge momentum in the field of cancer immunology both in terms of the rational design of

cancer "vaccines" and in the manner they would be administered clinically to patients. It is now believed that the immune system is not "actively" engaged in looking for abnormal tumour cells as was once thought, but instead is "ignorant" of them until an appropriate stimulus is received [48]. The critical factor therefore as to whether an immune response will be augmented against a particular antigen, is not whether it is "self" or "non-self" but instead whether an appropriate co-stimulatory signal is also expressed at the time of antigen presentation [48]. These appropriate or "danger signals" can arrive in multiple forms and include for example viruses, lipo-polysaccharide (LPS), cytokine release and necrotic cell death [49]. With respect to the latter it has been shown that DCs have the ability to take up necrotic as well as apoptotic bodies, however, only cells dying by necrotic means have the ability to stimulate DC maturation [49]. Perhaps the most notable members of this group, the heat shock family of proteins (HSPs) [50-54]. The HSP family of proteins have received a great deal of attention as key candidate molecules mediating the "danger signal". Heat shock proteins are discussed in greater detail by A. Menoret (Chapter 10) and consequently, only a brief overview of their function will be conducted here.

HSPs are molecular chaperones that have been implicated in a number of physiological processes ranging from protein folding and transport to the binding of intracellular peptides. Moreover, these proteins are considered as excellent candidates for mediating "danger signals" to DCs as they have an intracellular location under normal physiological circumstances and are only released upon cellular damage [50]. An increase in cellular stress is believed to result in the activation of HSP gene expression ultimately leading to an accumulation of protein [55]. HSPs have been shown to confer resistance to inflammatory cytokines such as TNF alpha and consequently they decrease a cell's likelihood of being killed by external agents [51]. In addition to having a direct effect upon cell survival, HSPs are also known to bind peptides that can be directly transferred to MHC class I molecules for antigen presentation [52, 56]. In a series of elegant studies Suto and colleagues were able to show that heat shock proteins complexed to antigenic peptides could be taken up by antigen presenting cells and transfer their peptides directly to MHC class I for T-cell presentation [57]. It was intimated therefore that in conditions of stress tumour cells might also release peptide bound HSPs that could be taken up by surrounding APCs for T-cell presentation. In-vivo tumour models have been extremely informative about the mechanism of HSP-peptide cross presentation to $CD8^+$ T-cells. In one specific example HSPs were isolated from syngeneic tumour cells and used in a prophylactic immunisation regime to protect against a lethal challenge of tumour cells. Protection occurred only when HSPs were peptide bound and that the peptides were derived from the same tumour type as the challenge. No protection was observed if the HSPs were stripped of tumour peptides, or if the peptides were administered in isolation [52]. This would support the contention that it was the peptide component that was providing a specific immune target for T-cell recognition and the HSP was critical for directing the peptide into the appropriate MHC trafficking pathways [52, 58]. In a recent finding by Todryk et al. inducing HSP 70 expression in tumour cells was found to lead to an increased infiltrate of macrophages, T-cells and DCs within the tumour mass and that immature DCs had the ability to

directly take up HSP/peptide complexes and present them to T-cells through cross priming mechanisms [51].

Heat Shock Proteins

It is suggested that heat shock proteins might represent one category of immune modulators possessing the requisite components for mediating "danger signals" to DCs possibly through antigen cross priming. This would not seem unreasonable since HSPs are known to bind peptides in a non-sequence dependent manner and are thus capable of providing an antigenic source. In addition it is now known that these proteins are taken into cells via receptor-mediated endocytosis which might suggest that a functional role in immuno-stimulation has evolved. It might also be implied that under normal physiological conditions, cell death via apoptosis would not lead to immune activation as apoptotic bodies are weakly immunogenic. This is clearly a favourable situation for the host organism and serves to attenuate immune activation for processes that are occurring constantly. If however, a cell becomes stressed due to a change in environmental conditions (e.g. anoxia) and induces the expression of HSPs to counteract these effects, apoptotic bodies produced from these cells (hence accumulating HSPs) would consequently be highly immunogenic. Conditions or stimuli producing large-scale apoptosis in the absence of HSP expression/inflammatory stimuli might also be a situation in which DC activation and maturation could occur.

Co-Stimulatory Molecules

The maturation of DCs is not simply confined to processes involved in optimal antigen presentation to T-cells. For effective T-cell stimulation to occur at the time of antigen presentation, it is necessary to have the simultaneous expression of several cell surface receptors or "costimulatory" molecules. In the absence of these co-stimulators, antigen presentation to T-cells is believed to result in either tolerance or clonal deletion [reviewed by 59]. A number of costimulatory molecules have been identified and their role in T/dendritic cell activation has been elucidated. They include for example B7, CD40 and OX40. While B7 has been extensively studied in relation to its ability to stimulate T-cell activity [reviewed by 60], it is the latter two receptor/ligand systems that have begun to generate a great deal of interest. These are discussed below:

CD40

The ability to cross prime antigens derived from exogenous sources is an extremely powerful means by which DC are able to present antigens to both $CD8^+$ and $CD4^+$ T-cells. Although CTL are the main effector cells for antigen recognition and killing, it has been demonstrated that to cross prime and activate $CD8^+$ T-cells, $CD4^+$ T-cell activation is mandatory [32, 37]. The co-operation between $CD4^+$ T-helper cells and $CD8^+$ CTL has now been shown to occur indirectly, via an intermediary activation of dendritic cells. In this model, antigen recognition by both T-cell subsets is believed to occur upon the same DC via activation of the CD40 receptor (a member of the TNFR super-family) thus obviating the need for direct $CD8^+/CD4^+$ interaction [61]. CD40L is

up regulated on activated T-helper cells and the binding of ligand to its cognate receptor on DCs is believed to be sufficient to "condition" the DC, promoting both maturation and presentation of co-stimulatory/ accessory molecules. In this state the DC is capable of directly priming naïve T-cells without further involvement of antigen specific T-helper cells [62]. Inhibiting CD40L interactions on DCs is sufficient to abolish CTL activation. The need for "specific" T-cell mediated help has been overcome through the use of antibodies that target CD40 receptors directly and it has been suggested that antibodies used in this manner could have significant value in the therapeutic treatment of cancer [63]. Ligation of the CD40 receptor is known to result in the production of several inflammatory cytokines including TNF-alpha, IL1-beta, IFN-gamma and IL-12. Except for IL-12, antibody depletion studies were shown to have little/no effect upon the DC maturation process and it was suggested that IL-12 release following CD40 ligation served to enhance DC maturation processes [64].

OX40

Another co-stimulatory receptor-ligand complex involved in T-cell activation by dendritic cells is OX40L (a member of the TNF family of proteins). OX40L is present on the surface of dendritic cells and binding to its cognate receptor on $CD4^+$ T-cells results in their cellular activation. Increased cytokine production for TNF alpha, IL-12, IL-1 beta and IL-6 have been reported [65]. In addition CD80 (B7.1), CD86 (B7.2), CD54 and CD40 expression were augmented to levels sufficient for T-cell activation [65]. The functional significance of up-regulating CD40 expression on DCs, would be to ensure maximal stimulation via CD40L and thus lead to full DC maturation. To gain a better understanding of OX40-OX40L interactions between T-cells and APCs, Gramaglia and co-workers engineered artificial APCs transfected with OX40L and/or B7.1 co-stimulatory molecules and studied T-cell proliferative responses. $CD4^+$ T-cells stimulated with antigen alone were able to produce OX40 for approximately two to three days post exposure after which a decline in receptor expression was noted. For APCs expressing OX40L in isolation, only a weak stimulation of IL-2 release was noted, however for $OX40L^+$ cells expressing the co-stimulatory molecule B7.1, $CD4^+$ T-cells were induced to proliferate and to secrete large amounts of IL-2. These authors suggested that OX40-OX40L interactions serve to prolong T-cell proliferation, enhance cytokine production and could even have a role in producing long-term memory $CD4^+$ cells [66]. OX40L has been implicated in a number of other roles including T-cell homing and B-cell activation. In a recent publication by Chen et al., [67] OX40L knockout mice were studied to address specific questions in relation to its function as a co-stimulatory molecule. OX40L -/- mice were deficient in contact hypersensitivity responses due an inability to correctly stimulate T-helper cells. These mice did not however, exhibit inappropriate T-cell homing or defective humoral immune responses. In-vitro, dendritic cells obtained from these knockout mice were unable to stimulate T-cell cytokine production and as a result OX40L was implicated as a co-stimulatory molecule necessary for dendritic/T-cell interactions. In parallel studies conducted by Kopf and colleagues [68], OX40 receptor knockout mice were generated and the formation of both extrafollicular plasma cells and antibody responses were measured. No perturbations were detected for either of these processes and consequently were

considered to be independent of OX40 involvement. The generation of primary and memory cytotoxic T-cells in response to viral infection were not affected either, however, the number of IFN-gamma producing $CD4^+$ T-cells was greatly reduced as were the number of $CD4^+$ cells infiltrating lung tissue following viral infection. The authors concluded that OX40 had a pivotal role in generating optimal $CD4^+$ responses in-vivo.

Th1 and Th2 cytokine release in response to DC maturation following antigen stimulation

The type of immune response elicited by a DC is of crucial importance in determining whether effective anti-tumour immune cells are mobilised [69, 70]. As outlined previously, DCs have the ability to take in, process and present antigens to both $CD4^+$ and $CD8^+$ T-cells through cross priming mechanisms. Following an up-regulation of requisite co-stimulatory molecules, T-cells become and activated and induced to proliferate in an antigen specific manner. What is becoming evident however, is that helper responses are not generic in nature, but are instead polarised between either Th1 associated cytokines such as IL-2, IL-12, IFN-gamma or Th2 associated e.g. IL-4 and IL-10. Th1 cytokines lead to the activation and proliferation of CTL, NK cells and the production of IgG2a isotype specific antibodies. In contrast, Th2 cytokines elicit B-cell activation and the production of IgG1 associated antibodies in the absence of substantial $CD8^+$ CTL activation. There is mounting evidence to suggest that the cytokine profile associated with a tumour might indeed have a strong bearing on the clinical outcome; this is considered in more detail in the context of experimental models in section 5 below.

In the clinical context, a similar trend has been bserved. For example, it was noted in 10 tumour biopsies taken from patients with SCLC, that high levels of IL-4, IL-6, IL-10 and TGF-beta1 were expressed in tumour infiltrating lymphocytes while IL-2 mRNA expression was low [70]. Th1 (IL-2, IFN-γ) and Th2 associated cytokine release (IL-4, IL-6 and IL-10) was measured from mitogen activated PBMCs derived from patients possessing bladder, prostate or renal cell carcinomas. It was found that the levels of Th1 cytokine secretion were drastically reduced in comparison to age matched controls and the suggestion was made that there is not a concerted shift from Th1 to Th2 but rather that the Th1 cytokine cascade is not functioning correctly [71]. The ratio of Th1:Th2 cytokine expression has also been determined in tumour samples from patients diagnosed with non-SCLC. It was found that a higher Th1 ratio was associated with patients with operable tumours while the converse was true for patients with recurring tumours [69].

Cancer patients often produce antibody responses against the tumour and this has often been looked upon as a favourable sign, but other authors would suggest that an antibody response might actually favour tumour growth [72]. With a deeper understanding of how cytokines polarise immune responses, one might envisage a scenario in which the activation of Th1 associated cytokines is mandatory for the effective eradication of tumours. Shifting the balance towards Th2 and therefore predominantly an antibody response would ultimately result in the attenuation of CTL

and NK responses. If Th2 responses favour tumour growth, one might even speculate that the ability to actively subvert the immune system towards a Th2 immune response could indeed provide a distinct survival advantage for a tumour.

Summary of DC properties

To elicit effective ant-tumour responses it is necessary to activate and mobilise T-cells capable of recognising tumour-associated antigens. In order to achieve this goal, co-stimulatory molecules must be present on DCs at the time of antigen presentation as these receptors serve to enhance T-cell function and thus home their ability to recognise and kill target cells. CD40/CD40L and OX40/OX40L interactions serve to enhance the maturation of DCs making them effective antigen-dependent stimulators of $CD8^+$ T-cells. In this context, T-cells are activated and not tolerised to MHC restricted tumour antigens. Another important consideration is not simply whether an immune response is elicited against a tumour antigen but whether the immune response is primarily CTL and not antibody based. For this to occur therefore, it is necessary to favour the development of Th1 cytokine cascades (IL-2, IL-12, IFN-γ) over those stimulating T-helper cells to release Th2 associated cytokines (IL-4, IL-5, IL-10, and IL-13).

Type 1 and Type 2 tumour immune responses

It is now generally accepted that both T helper (Th) cells and T cytotoxic (Tc) cells can be segregated into two general categories based on their cytokine release patterns [73-76]. A type 1 cell (T1) selectively secretes IL-2, IFNγ and TNFβ/LT, whereas type 2 cells (T2) secrete IL-4, 6, 9 and 13. IL-5 and 10, generally thought of as T2 cytokines can be more promiscuous and also found associated with cells of a T1 phenotype. All of these cells appear to share a common precursor that can differentiate along either pathway. The final pathway is determined by the cytokine milieu in which the T cells are activated and undergo differentiation [77]. The presence of T1 or T2 cytokines, will drive uncommitted T cells to develop a cytokine profile similar to that which they are exposed, while at the same time inhibiting the development of cells with the reciprocal phenotype. Thus, IFNγ selectively expands T1 cells and inhibits proliferation of T2 cells, while IL-4 and IL-10, can selectively inhibit cytokine secretion by T1 cells [75, 78, 79]. This ability to inhibit the maturation of cells producing cytokines of the alternate type may account for the tendency to see a predominant cytokine profile. Therefore, the presence of T2 cytokines during the initial interaction between the T cell and tumor antigen presented by APC in the draining lymph node would facilitate the development of a T2 antitumor response. This could be caused by secretion of T2 cytokines by tumor cells, by secretion of IL-4 by NK1.1 T cells, or by IL-6 secreted from APC, [75, 80-84]. The secretion of IFNγ by NK cells or IL-12 by APC acts reciprocally and would be expected to activate T1 and inhibit T2 cells [75, 84-86].

Exposure to antigen in the absence of costimulation also induces T2 responses [87, 88]. This in turn blocks the release of IL-12 by dendritic cells, inhibits production of T1 cells and allows T2 responses to be established. The dose of antigen used to

sensitize T cells also affects the type of cytokine response. High concentrations lead to a predominant T1 response in CD4 cells, whereas low doses of antigen promote differentiation of predominantly T2 cells that produce high levels of IL-4 [reviewed in 87, 89]. A T2 profile also may develop as the default response if antigen is presented to T cells in the absence of inflammatory cytokines.

The importance of T1 or T2 responses in the pathogenesis and control of a number of diseases has been reviewed [75]. T1 responses protect or cure animals infected by protozoa, bacteria or fungi and appear to cause the destruction observed in transplant rejection and in the following autoimmune diseases: EAE, multiple sclerosis, psoriasis vulgaris, insulin-dependent diabetes mellitus, and rheumatoid and reactive arthritis. In contrast, T2 responses appear to be important in models where helminths are studied, and causative for allergic reactions, including atopic asthma and Omenn's syndrome.

The differential effects of T1 and T2 responses in tumor-bearing animals has only been appreciated recently. [90, 91]. Aruga et al demonstrated that the efficacy of T cells in an adoptive transfer model could be abrogated by neutralizing Mabs to IFNγ and GM-CSF, and augmented by treatment with anti-IL-10 [91]. Examining the effect of IL-12 administration on cytokine production at the tumor site in the same murine tumor model found that tumors from untreated mice had a rim of CD3+ T cells associated with production of IL-2, 4 and 10 (T2 response), but no IFN-γ [90]. However, tumors from IL-12 treated mice, exhibited high levels of IFN-γ production (T1 response) while IL-2, 4, and 10 production was decreased. This suggests that the balance between the production of T1 and T2 cytokines is a key determinant of whether or not a therapeutic effect is seen.

Using a B-cell lymphoma model, Lee and colleagues have reported that a cytotoxic T-cell immune response could be elicited in animals susceptible to tumour growth and that the response was rapid, being detected as early as 4 days post-challenge. The difference between resistant and susceptible animals was attributed to the type of helper responses being produced in the former group. It was found that Th1 cytokines were preferentially being secreted and that CTL development was stronger and earlier in the resistant group of animals [92]. It is known that antigen presentation can occur through DCs, macrophages or B-cells and the extent to which one particular population of cells might be recruited to present antigen has been speculated to play a role in the process of tumour progression. Qin et al. have reported that animals devoid of B-cells were resistant to tumour challenge and that their re-introduction resulted in sub-optimal helper responses for stimulating CTL activity.

This concept is supported by two further clinical studies in which T1 responses were associated with tumor regression. Kawakami et al, noted higher response rates in patients treated with tumor-infiltrating lymphocytes (TIL) exhibiting gp100-specific IFN-γ production, and Lowes et.al. demonstrated that mRNA levels for T1 cytokines were significantly higher in spontaneously regressing melanomas compared to progressing lesions [13, 93]. These data support the hypothesis that induction of a T1 response is necessary for tumor regression and that type 2 responses are either ineffective or potentially harmful because of their ability to inhibit T1 responses.

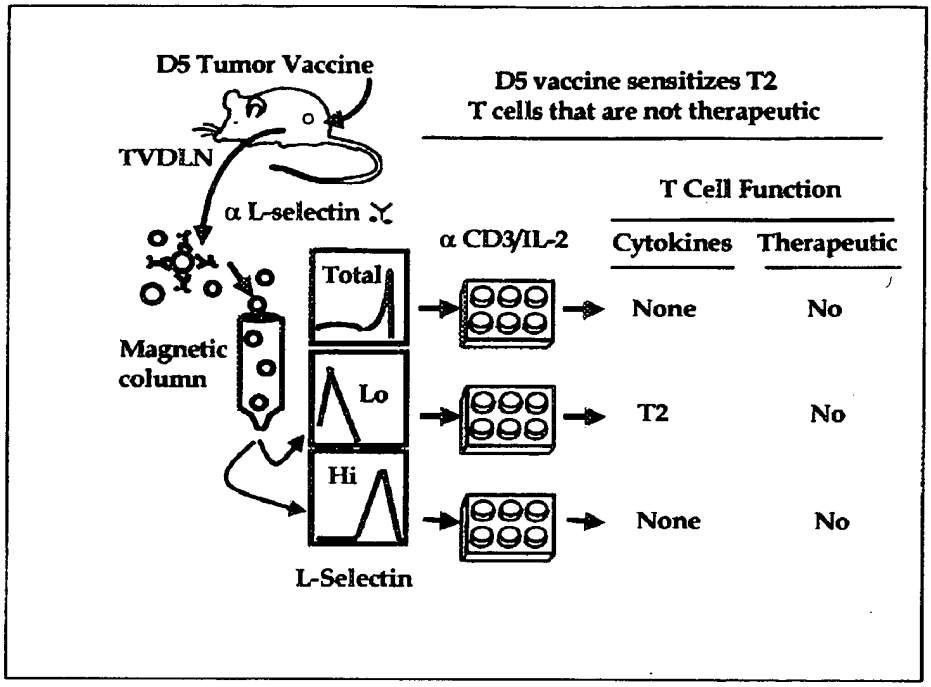

Figure 1.1. Day 7 D5-TVDLN were harvested and T cells isolated and labeled with anti-L-selectin magnetic beads. T cells were separated by passage over a magnetic column, into populations expressing low or high levels of L-selectin. These cells were activated with anti-CD3 for 2 days and expanded in low dose IL-2 for 3 more days. T cells were then assayed for tumor-specific cytokine secretion and adoptively transferred into mice bearing 3 day established pulmonary metastases.

However, it is generally accepted that the failure of vaccination with poorly/non immunogenic tumors to protect the host from a subsequent tumor challenge is because the host fails to generate an antitumor immune response.

Recent data from Hu and colleagues demonstrate that this scenario is not true for the poorly immunogenic B16BL6-D5 tumor. They used a method pioneered by Kagamu et al., to isolate tumor-reactive T cells [94]. This method exploits the observation that T cells responding to antigen in tumor vaccine draining lymph nodes (TVDLN) will down regulate expression of L-selectin (CD62L), a well established marker of recently activated T cells [95-99]. Kagamu isolated T cells with reduced expression of L-selectin (L-selectin^{LO}) from lymph nodes draining an immunogenic sarcoma and documented that all the therapeutic activity was confined to the T cells with the L-selectin^{LO} phenotype [94].

Based on these studies, Hu and colleagues examined lymph nodes draining the poorly immunogenic melanoma, B16BL6-D5 (D5), and observed reduced expression of L-selectin, suggesting that T cells were responding to the tumor vaccine [100]. They then

used the same approach used by Kagamu et al., to see if the enriched population of potentially tumor-reactive T cells would mediate tumor regression in an adoptive transfer model (Figure 1.1).

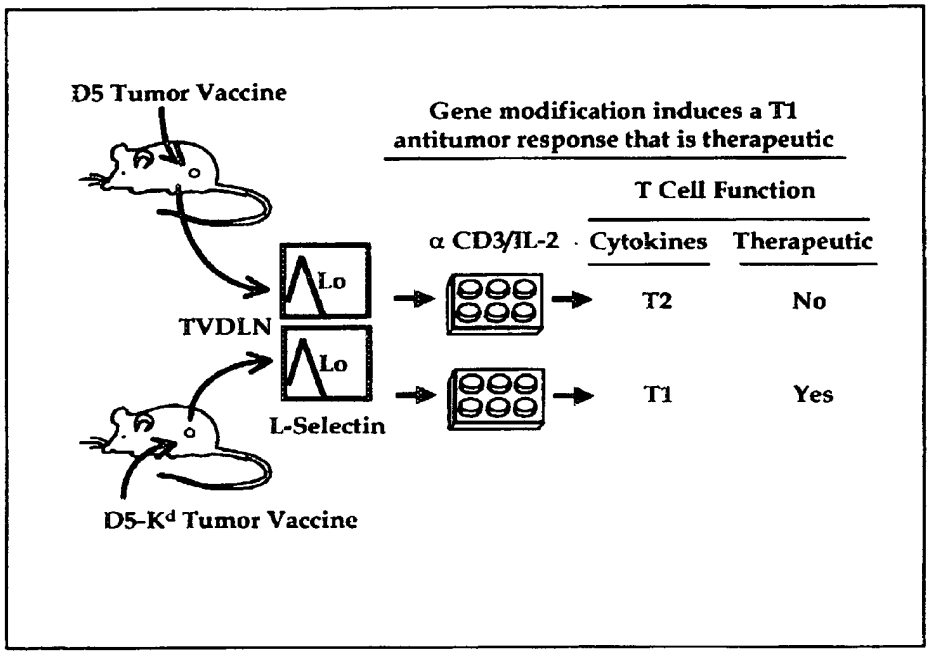

Figure 1.2. Day 7 TVDLN were harvested from mice vaccinated with either D5 or the allo-modified (D5-K^d) vaccine and T cells were separated, by passage over a magnetic column, into populations expressing low levels of L-selectin (L-selectin^{LO}). These cells were activated with anti-CD3, expanded in IL-2, and assayed for antitumor activity as described in figure 1. Effector T cells generated from the D5 vaccine exhibited a tumor-specific T2 cytokine profile and were non therapeutic, while effector cells generated from the D5-K^d vaccine exhibited a tumor-specific T1 cytokine profile and mediated tumor regression.

Although L-selectin$^{Lo/-}$ T cells were not therapeutic in vivo, they exhibited a strong tumor-specific cytokine response to stimulation with D5 tumor in vitro; the dominant cytokines produced were type 2; IL-4 and IL-10. These results demonstrated that T cells in lymph nodes draining the poorly immunogenic tumor were not ignoring the tumor, but were specifically responding to tumor with a T2 cytokine response. The reason that this observation had not been made previously probably relates to the requirement to enrich for tumor-specific (L-selectin^{LO}) T cells in order to identify the low level of tumor-specific IL-4 secretion.

Hu and colleagues, then directly compared the L-selectin^{LO} TVDLN from mice vaccinated with both the non therapeutic (D5) and the therapeutic (D5-K^d) vaccine [100]. T cells from either vaccine were phenotypically identical, but while non therapeutic T cells exhibited a tumor-specific T2 response, therapeutic T cells made a T1 response (Figure 1.2).

While these data support the hypothesis that immune deviation occurs following exposure to D5, they do not offer proof that immune deviation is responsible for the

failure of vaccination. To directly test this possibility they next examined whether it was possible to polarize D5 primed TVDLN away from a T2 profile and towards a T1 cytokine profile. Adding neutralizing anti-IL-4 Mab and a source of IL-12 to the culture media during the in vitro activation and expansion of D5 TVDLN T cells they were able to polarize tumor-specific T cells to a T1 cytokine profile (Hu et al., manuscript in preparation). Coincident with this "repolarization" the T1 T cells aquired therapeutic activity while the tumor-specific T2 cells did not (Figure 1.3).

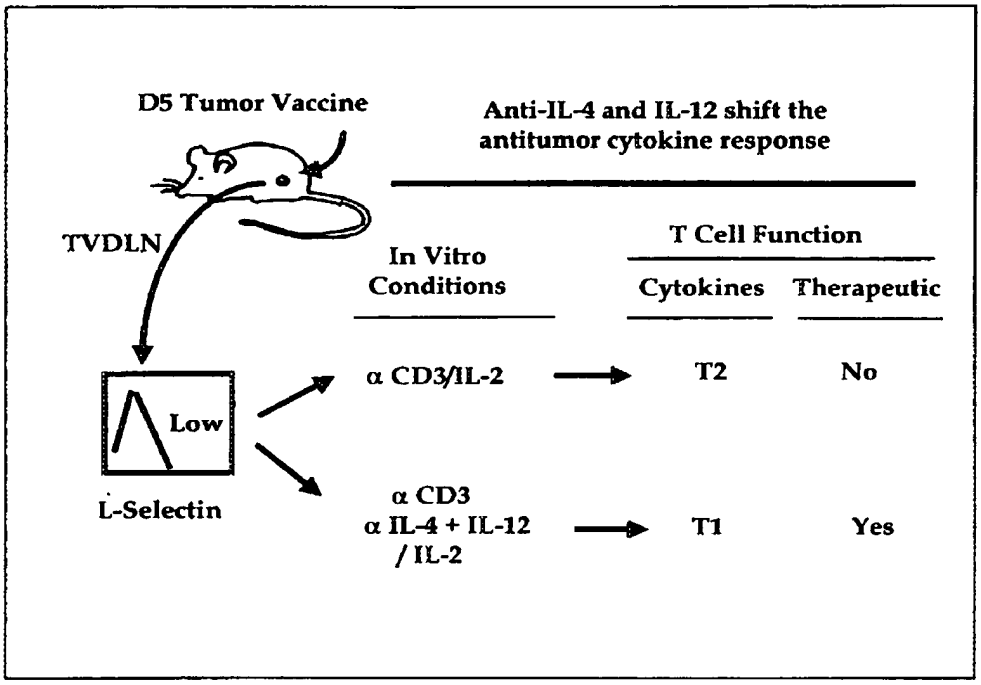

Figure 1.3. L-selectin^{LO} T cells were isolated from day 7 D5-TVDLN. Half the cells were cultured as described above and the remainder were cultured similarly, but with the addition of a neutralizing anti-IL-4 and a source of IL-12 during the anti-CD3 activation step. While the standard anti-CD3 and IL-2 culture generated T cells with a tumor-specific T2 cytokine response that were not therapeutic, the T1 promoting culture generated T cells with a dominant tumor-specific T1 cytokine response that mediated significant tumor destruction.

These results beg the question: Do animals that are vaccinated with other poorly/non immunogenic tumors recognize them and make a T2 immune response that is non destructive? Do immunogenic tumors prime a T1 response? Before you can ask this question it is important to have a standard definition and understanding of the term "immunogenicity". In tumor models, immunogenicity is functionally defined by whether vaccination with irradiated unmodified tumor provides protection from a subsequent challenge with a dose of viable tumor cells that will uniformly generate

tumors in naïve mice. Since the dose of tumor cells used for vaccination can make a major difference in the efficacy of the vaccine, 10^7 cells given 10,000 rads are routinely used [100]. Since the timing and dose of tumor cells used to challenge can substantially effect vaccine efficacy, mice are generally challenged with two to five times the TD100 (lowest dose of cells that form tumors in 100 % of naïve animals) 14 days following vaccination. Using this method, vaccination with poorly immunogenic or non-immunogenic tumors will not protect any animals from a tumor challenge, while vaccination with weakly immunogenic tumors will protect 20-40 percent of animals from a tumor challenge. In contrast, vaccination with strongly immunogenic tumors will uniformly protect 90 to 100 percent of animals from a tumor challenge.

To determine how generalizable this T1/T2 paradigm might be for predicting immunogenicity, a panel of murine tumors were analyzed to see if a correlation could be drawn between induction of a T1 response to vaccination and protection from a tumor challenge. The data presented in table 1.1 summarizes a series of experiments and supports the hypothesis that poorly/non immunogenic and weakly immunogenic tumors induce either a T2 or a mixed T1-T2 response while strongly immunogenic tumors induce a dominant T1 response (Winter et al., manuscript in preparation).

While these preliminary findings document a strong correlation between induction of a T1 antitumor immune response and vaccine efficacy, others have documented T2 effector mechanisms that can mediate tumor regression [101-103]. Clearly, additional studies will need to evaluate how these tumor-specific T2 cytokine responses, generated in mice with cytokine transduced tumor vaccines or using TCR transgenic T cells, will compare to the correlation we have made between the primary response to tumor vaccination and the ability to induce protective immunity. It is likely that different mechanisms will mediate tumor regression in vaccine challenge experiments and adoptive transfer studies. Our own studies document a reliance on IFN-γ in vaccination challenge experiments with the D5 tumor, but additional T1 cytokines appear to compensate, in the absence of IFN-γ, and mediate tumor regression in adoptive transfer studies using the same tumor (Winter, Hu and Fox manuscript in preparation). These findings should not be viewed as controversial, but as a compliment to the diversity of the immune response, particularly as it relates to tumor immunity.

Unlike the rather straightforward T1/T2 paradigm seen in infectious disease models, the preliminary findings in tumor models infers that a more complicated series of effector mechanisms exist. Even vaccines that normally induce strong T1 responses can not do so in a vacuum: Development of an effective antitumor T1 cytokine response has been shown to require some IL-4 during the initial priming of the immune response [104].

Another potential problem with the hypothesis that T1 cytokine responses are effective at mediating tumor destruction lies in defining the mechanism of tumor destruction. While not ruling out the role for cytokines, conventional wisdom has long held that for most T cell-mediated mechanisms, tumors are destroyed by cytolytic T cells. Since the two principal methods of cytolysis are via perforin or Fas/FasL [105], Winter and colleagues examined whether tumor-specific T1 T cells, from animals deficient in either of these two lytic mechanisms, could mediate tumor regression. Their

results clearly show that T cells from perforin knock-out (PKO) or FasL mutant (gld) animals can mediate highly therapeutic antitumor activity (Figure 1.4)[106].

Table 1.1 Tumor-specific T2 cytokine profile of TVDLN effector T cells predicts vaccine failure.

Tumor TVDLN	Strain	Histology	Immuno-genicity	Cytokine profile
B16BL6-D5	C57BL/6	melanoma	non/poor	T2
MPR-5	C57BL/6	prostate	non/poor	T2
4T1	BALB/c	breast	non/poor	T2
MCA-310	C57BL/6	sarcoma	weak	T2=T1
MCA-304	C57BL/6	sarcoma	strong	T1
MCA-309	C57BL/6	sarcoma	strong	T1

While these studies rule out a critical role for either cytolytic effector mechanism in the B16BL6-D5 melanoma model, it is possible that compensatory mechanisms may allow FasL to be more active in PKO mice and vice-versa in gld mice, or that other effector mechanisms exist. To further examine this possibility Yamada has developed multiple gene knock-out animals to further examine this hypothesis (Yamada and Fox unpublished observation).

Considering these findings, Schwartz's definition of tolerance as it relates to patients with cancer might be better stated as follows: "a physiological state in which the immune system does not react destructively against cancer". We have provided evidence that the "non immunogenic" D5 tumor does sensitize tumor-specific T cells in vivo; however, because they produce T2 cytokines, tumor cells are not destroyed. The allo-modified or GM-CSF secreting vaccine induces T1 T cells that can mediate therapeutic activity in adoptive transfer studies.

These observations clearly identify immune deviation, a mechanism of tolerance, as being operational during the initial immune response following vaccination in the D5 tumor model and suggest possible sites to examine in patients with cancer. Recent studies suggest that separation strategies that exploit reduced L-selectin expression are useful in analyzing TVDLN of patients on vaccine trials [107]. Careful examination of a patient's initial immune response to vaccination could be predictive of a vaccines therapeutic potential and may provide a platform for the design and monitoring of a new generation of clinical trials.

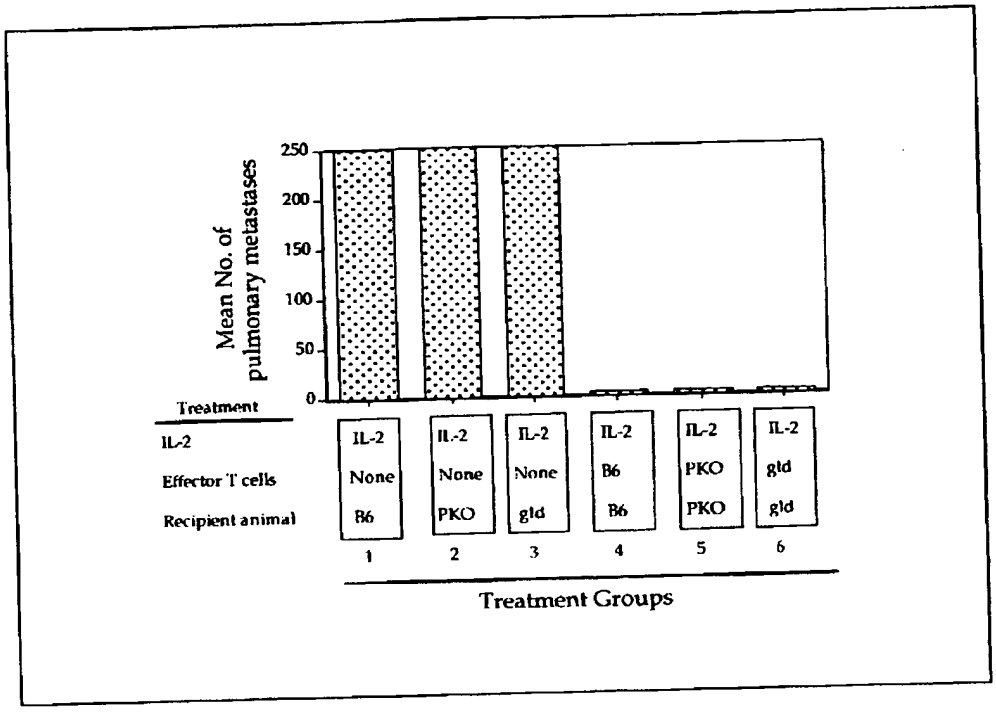

Figure 1.4. C57BL/6 (B6), perforin knock out (PKO) or FasL mutant (gld) mice were vaccinated with a GM-CSF producing D5 tumor (D5-G6) in order to generate effector T cells with a tumor-specific T1 response. B6, PKO and gld mice bearing 3 day established pulmonary metastases were treated with either IL-2 alone (Groups 1-3, 90,000 IU bid x 4 days) or with IL-2 and $7x10^7$ effector T cells, matched for the same genotype (groups 4-6, B6, PKO, gld). Similar results were obtained in two additional experiments.

IMMUNOTHERAPY TREATMENT OF CANCER PATIENTS USING DC BASED VACCINES

There is now a concerted effort to use immunological based vaccines for the treatment of cancer due to the inefficiency and toxicity problems that are associated with conventional treatments. The number of clinical trials involving DCs is increasing steadily and the approaches being used are varied. For example in the treatment of prostate cancer, phase I clinical data has been published regarding the efficacy of dendritic cell based vaccines pulsed with peptides from the prostate specific membrane antigen (PSMA). The patients (who were clinically refractive to hormone ablation therapy) were divided into groups receiving either peptide, DC or DC pulsed with peptide. Clinical responses (as measured by a reduction in the level of prostate specific antigen (PSA)) were detected only in the group receiving DC pulsed with PSMA peptides [108]. In a follow up study, patients responding to treatment were monitored

closely for a variety of prostate markers including PSA, free PSA, PSMA and alkaline phosphatase. Many of these patients were still found to be clinically responsive to treatment 200 days post vaccination, suggesting that treatment was long lived and contingent upon the presence of DCs for effective anti-tumour responses [109, 110]. In order to determine whether GM-CSF might increase the immunogenicity of DCs pulsed with PSMA, patients were also given co-administrations of the cytokine; no augmentation in immune response was detected [111]. In a similar study Nestle and co-workers pulsed autologous DCs (obtained from patients diagnosed with melanoma) with peptides from known melanoma associated antigens. Keyhole limpet haemocyanin was added as a $CD4^+$ helper antigen and immunological tracer molecule. Objective responses were detected in 5 out of 16 patients – two showed complete whereas three patients had partial responses and regression of metastasis was noted in the skin, soft tissue, lung and pancreas. One patient was noted as having a minor response during the course of therapeutic treatment. No side effects or autoimmunity was detected from any of the patients [112]. Dendritic cell immunotherapy has not been restricted to solid tumours. DCs from patients diagnosed with follicular B-cell lymphomas were pulsed with a tumour specific idiotype protein. Although the number of patients involved in the study was limited (4), all patients were able to mount detectable anti-tumour immune responses. One patient had complete tumour regression, one with partial and a third resolved all evidence of disease as measured by molecular assays [113].

Future of cancer immunotherapy using dc based vaccines

There is no doubt that the number of clinical trials based upon DC vaccine technology will increase exponentially within the next five years. As more is learned with respect to costimulatory molecules (e.g. B7, CD40, OX40 etc) it is likely that DCs will be "appropriately primed" (e.g. via CD40 ligation) for maximum antigen presenting and T-cell stimulatory capacity. The feasibility of this idea has already been tested in in-vivo animal models as highlighted by the work of Diehl et al. These authors noted that animal immunisations regimes consisting of peptide alone, resulted in T-cell tolerance whereas the addition of activating antibodies against CD40 in combination to a CTL specific antigen was sufficient to change a tolerance signal to a $CD8^+$ T-cell stimulatory signal. It was suggested that this type of approach might be an effective way in which to stimulate an immune response against pre-established tumours [114]. The idea of stimulating an appropriate T-helper response in parallel to providing specific CTL epitopes is gaining such momentum that MHC class II binding epitopes from known tumour antigens are now being actively sought. To date several have been discovered and it is likely that they will be used as part of an immune "adjuvant" for stimulating a maximal $CD8^+$ T-cell response against tumours [115]. An alternative approach for activating T-helper cells was developed by Wu et al., who used DNA targeting constructs to take endogenously synthesized antigens directly into the class II processing pathways for presentation to $CD4^+$ T-cells [116, 117]. DNA based vaccines represent a powerful tool in the fight against cancer since they can be produced and administered to patients relatively easily and once constructed, DNA vaccines are known to have a uniform and consistent quality. DNA "minigene" vaccines have received a great deal of attention

because they code only for MHC class I CTL epitopes [118-122]. Several epitopes can be arranged in a linear manner to produce a single protein and this has been shown to be an extremely effective mechanism for inducing anti-tumour immunity. This immunity is likely to work through either direct or indirect transfection (e.g. taking up of apoptotic bodies from transfected cells) of DCs. One might envisage a scenario in which haplotype specific "minigenes" for a given tumour antigen is given in combination with MHC class II encoding DNA vaccines to produce maximal effect. DCs have been shown to release vesicles commonly referred to as "exosomes", that contain MHC class I, MHC class II and co-stimulatory molecules. Antigen specific peptide pulsing of "exosomes" resulted in the formation of CTL clones followed by the suppression or eradication of pre-established tumours [123]. While the ease and reproducibility of "exosome" preparations for tumour immunotherapy is questionable, it has stimulated the idea of creating artificial liposome structures that possess "appropriate" co-stimulatory molecules necessary for maximising T-cell activation. If this could be achieved, it would circumvent problems associated with isolating host specific DCs, quality of preparation, reproducibility and cost since these molecules could be manufactured consistently on a large scale. Another area receiving attention is the development of HSP based immunological vaccines that carry tumour antigens into DCs. As outlined previously heat shock proteins are known to bind peptides in a relatively non-specific manner thus making them superb candidates as immune adjuvants for dendritic cells. There is a lot of supporting evidence that these molecules could have a dual function in not only carrying peptides directly into MHC class I loading pathways but also in acting as an immune adjuvant ("danger signal") boosting CTL responses against tumours.

SUMMARY

If "danger signals" are found to be absolutely necessary for successful immune priming against tumours then it is likely that vaccination strategies must encompass the use of both MHC class I and class II targeted epitopes in conjunction with a potent immune adjuvant. MHC class II binding epitopes would be mandatory for stimulating optimal $CD4^+$ T-cell activity, concomitantly leading to full CTL maturation. In addition, co-stimulatory molecules are fundamental in the antigen priming process and it is likely therefore, that an appropriate immune adjuvant would have to be employed in order to ensure maximal expression at the time of antigen presentation to T-cells. In the absence of co-stimulation, peptide presentation would almost certainly result in tolerance rather than activation. It is possible therefore that a survival advantage would ensue if tumours had the ability to present antigens to T-cells in the absence of co-stimulatory molecules. This would serve to promote T-cell tolerance and prevent the activation of an effective immune response against the tumour. One might envisage a scenario therefore, in which immunisation regimes would be administered to patients on a regular and consistent basis and thus help to overcome any tolerising activity initiated directly from the tumour.

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